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Molecular Mechanism of Bacterial Magnetite Formation and Its Application

Tadashi Matsunaga and Yoshiko Okamura
Department of Biotechnology, Tokyo University of Agriculture and Technology
Naka-Cho, Koganei, Tokyo 184-8588, Japan

ABSTRACT

Nano-technology has been identified as an area which will bring about new evolutions in materials, devices and processes. The challenges of nano-biotechnology entail manufacturing more sophisticated and highly efficient biosensors and biomaterials at the nano-scale level for use in interdisciplinary fields. Here, we introduce a biomaterial produced by magnetic bacteria, bacterial magnetic particles (BMPs), and the molecular architecture technique we have used for its application.

Magnetic bacteria synthesize intracellular magnets which are encapsulated by lipid bilayer membranes. Sizes of BMPs vary from 50 - 100 nm in diameter, and number over 10 per cell. BMPs are composed of magnetite (Fe_3O_4) with a single magnetic domain. Easy aqueous dispersion of BMPs enable development of highly sensitive chemiluminescence enzyme immunoassays by the chemical coupling of antibodies on BMP surfaces. BMPs can likewise be used as drug delivery systems employing magnetoliposomes with high capture volumes. We previously reported a technique for preparing recombinant BMPs on which proteins were displayed by gene-fusion. We furthermore applied such recombinant BMPs to biotechnologically-important issues, including novel bioassay platforms for medicine and environmental management. We envisage the production of more refined chemicals and agents through expression on BMPs by gene-fusion followed by simple purification using magnet.

INTRODUCTION

The use of magnetic particles in immunoassays enables the separation of bound and free analytes by applying a magnetic field. For example, proteins can be attaching covalently to solid supports, such as magnetic particles, preventing the desorption of antibodies during assaying. Because these particles disperse evenly throughout the reaction mixture, they allow rapid reaction kinetics without the need for continuous mixing or shaking, enable coupling antibodies, and facilitate ease of use. Magnetic particles serve as both solid supports and a means of separation in the assay system.

Magnetic bacteria have been isolated from freshwater and marine sediments, and are known to produce magnetite particles which are aligned in chains of around 20 particles per cell [1-4]. Bacterial magnetic particles (BMPs) are nano-size and disperse well in aqueous solution due to their stable lipid membranes [5]. On the basis of these properties, BMPs have been utilized in fluoroimmunoassays [6-8], mRNA recovery [9] and as DNA carriers [10]. Because of their fastidious characteristics and undefined metabolism, pure cultures of magnetic bacteria are very difficult to achieve. However, the successful isolation, cultivation and colony formation of the magnetic bacteria, *Magnetospirillum magneticum* AMB-1 [3] and MGT-1 [2], which are tolerant to oxygen, have allowed development of conjugative gene transfer and transposon mutagenesis systems [11]. Several recent findings and the mechanism of magnetite particle formation have been ascertained through studies with AMB-1. Thus, we describe here the molecular, genetic and biotechnological characteristics of magnetic bacteria and bacterial magnetic particles, revealed mainly from our recent studies with respect to the

genetic engineering of magnetic bacteria and advanced applications of bacterial magnets based on molecular architecture.

MAGNETIC BACTERIA

Magnetic bacteria are a heterogeneous group of gram-negative prokaryotes with both diverse morphologies and habitats [12-20]. Various morphological types of magnetic bacteria including cocci, rods, vibrio, and spirilla have been isolated from sediments, *e.g.* marine, rivers, lakes, ponds, beaches, rice paddies, drains, wet soil [21], deep sea [22] and estuaries. Most magnetic bacteria produce nano-sized magnetite with species- or strain- specific morphologies [23-26]. The alignment of BMPs in chains confers a magnetic dipole and magnetotaxis to cells allowing bacterial orientation along geomagnetic field lines. The successful isolation and cultivation of a facultative aerobic magnetic bacterium, *Magnetospirillum magneticum* AMB-1 (Fig.1a), has enabled us to conduct genetic manipulations to elucidate the mechanism of BMP formation, and to optimize culturing for industrial-scale production of BMPs. We have also isolated a novel sulfate-reducing magnetic bacterium, *Desulfovibrio magneticus* (sp. nov.) (Fig.1b), formerly known as RS-1, which grows and synthesizes BMPs under strict anaerobic conditions [4].

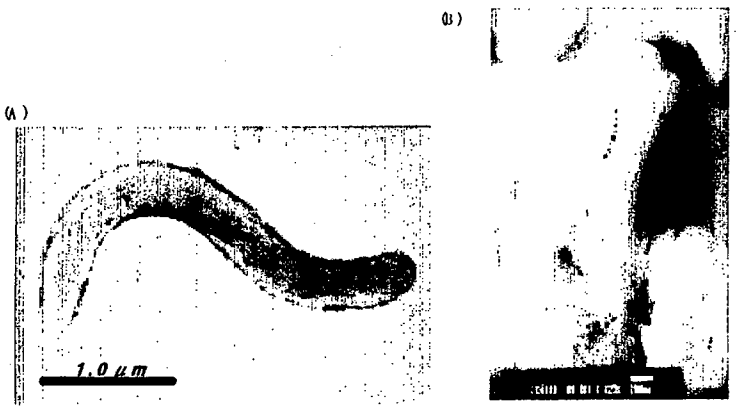

Fig. 1 Transmission electron micrographs of *Magnetospirillum magneticum* AMB-1 (A) and *Desulfovibrio magneticus* RS-1 (B).

BACTERIAL MAGNETIC PARTICLES

Transmission electron microscopy shows BMPs to be encapsulated by a biomembrane (Fig.2). The membrane is approximately 2-4 nm in thickness. Analysis of membrane components has been carried out mainly on strain AMB-1. Results indicate that BMP membrane consists of lipid containing phospholipids (comprising 58-65% of the total lipids) of which about 50% contains phosphatidylethanolamine [6, 27-28]. Moreover, the fatty acid make up of BMP membrane is similar to the cytoplasmic membrane in strain AMB-1. Almost all of the proteins expressed in the cytoplasmic membrane were also found in the BMP membrane. However, five proteins were shown to be specific to BMP membrane fractions. [29, 30].

Fig. 2 Bacterial magnetic particle from *Magnetospirillum magneticum* AMB-1.

GENETIC ANALYSES OF *MAGNETOSPIRILLUM MAGNETICUM* AMB-1

Isolation and characterization of the genes that mediate magnetite formation in bacteria are prerequisite for determining the mechanisms of magnetic particle biosynthesis. In order to identify specific genes involved in magnetite synthesis, transposon mutagenesis was conducted with strain AMB-1 [11]. Numerous non-magnetic mutants were successfully obtained and *magA* gene was subsequently isolated from one of the numerous mutants (strain NM5) by analyzing of the transposon disrupted gene (Fig. 3). The *magA* gene has homology with the Na^+ / H^+ antiporter, NapA, from *Enterococcus hirae*. The iron up-take activity of MagA protein was determined using inverted vesicles prepared from fragmented membrane expressing MagA protein in *Escherichia coli* (Fig. 4). Addition of ATP initiated accumulation

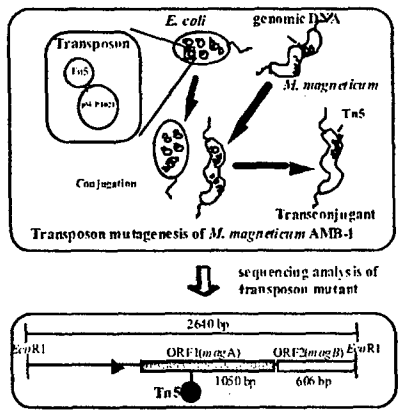

Fig. 3 Transpospn mutagenesis of *Magnetospirillum magneticum* AMB-1 and isolation of *magA*.

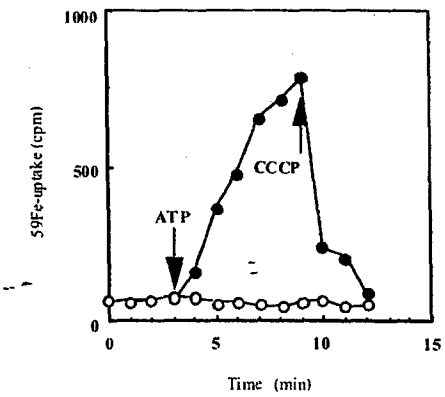

Fig. 4 Iron uptake into inverted vesicles. Arrowhead indicated the point of addition of ATP or CCCP.
● ; the vesicles expressed MagA
○ ; control

of ferrous ion in vesicles and the ion was released by adding of carbonyl cyanide m-chlorophenylhydrazone (CCCP) known as protnophore. The activity was also observed under artificial proton gradient without ATP. These results suggests that MagA protein is a proton-driving H^+ / Fe(II) antiporter [31]. Additionally, intracellular localization of the MagA protein was examined using a MagA-Luciferase fusion protein, indicating that MagA is localized on both the cytoplasmic and BMP membranes, where it appears to transport iron into BMP vesicles [32]. Therefore, MagA is considered to play an essential role in the bacterial formation of magnetite crystals.

Presupposing that proteins expressed on BMP membranes play a direct role in regulating magnetite crystallization, a number of distinct BMP-specific proteins have since been identified. Protein fractions prepared from cellular and BMP membranes and cytoplasm were separated by SDS-polyacrylamide gel electrophoresis, reveal five specific proteins in BMP membrane fractions (Fig. 5). Utilizing 2D-electrophoresis, 4 of these proteins were separated for amino acid sequencing. On the basis of the N-terminal amino acid sequences determined, oligonucleotide primers were designed to perform polymerase chain reactions (PCR) to amplify DNA sequences of the target proteins. Subsequently, *mpsA* encoding a 36 kDa protein and *mms16* encoding a 16 kDa protein were isolated. DNA sequences of these two genes were analyzed and protein homology was examined. The amino acid sequence of MpsA was

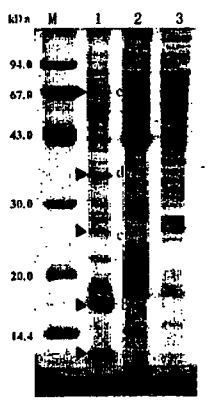

Fig. 5 Protein profiles by SDS-PAGE. 40μg of protein from each fraction was applied. Lane 1: BMP membrane solubilysate ; lane 2: membrane solubilysate : lane 3: cytoplasmic fraction ; M: standard markers.

proven to be highly homologous with that of *E. coli* acetyl-CoA carboxylase [29], and the *mms16* gene encodes for a GTPase [33]. Mms16 protein was found to be the most abundantly expressed of the five BMP specific proteins. We hypothesize that Mms16 is a GTPase with properties similar to eukaryotic small GTPases which control vesicle trafficking. Thus, we conducted inhibition experiments on GTPase by aluminum fluoride. AlF_4^- prevents BMP synthesis suggesting that GTPase activity is required for BMP synthesis [33]. Thus, we hypothesize that magnetosomes arise through invagination of the cytoplasmic membrane and processes similar to those of eukaryotic vesicle formation.

PROPOSED MECANISM FOR MAGNETITE FORMATION

We have hypothesized that the BMP membrane is derived from the cytoplasmic membrane and formed through the invagination process. Transmission electron microscopy of *M. magnetotacticum* MS-1 reveals that BMP envelopes appear prior to the crystallization of magnetite [28]. The mechanism of envelope formation, however, still remains unclear. Previous studies on vesicle formation by invagination of the cytoplasmic membrane have been performed using eukaryotes. It is possible that magnetic bacteria have similar mechanisms of vesicle formation. We propose that Mms16 mediates the priming of invagination and MpsA mediates the acylation of Mms16 to anchor the BMP membrane. Moreover, MagA on BMP membranes, transfers Fe ions into intracellular vesicles. Various proteins associated with the BMP membrane may play functional roles involved in magnetite

generation inside BMP envelopes. These include: 1) the accumulation of supersaturating iron concentrations and maintenance of reductive conditions; and 2) the oxidation of iron to induce mineralization, or the partial reduction and dehydration of ferrihydrite to magnetite (Fig. 6).

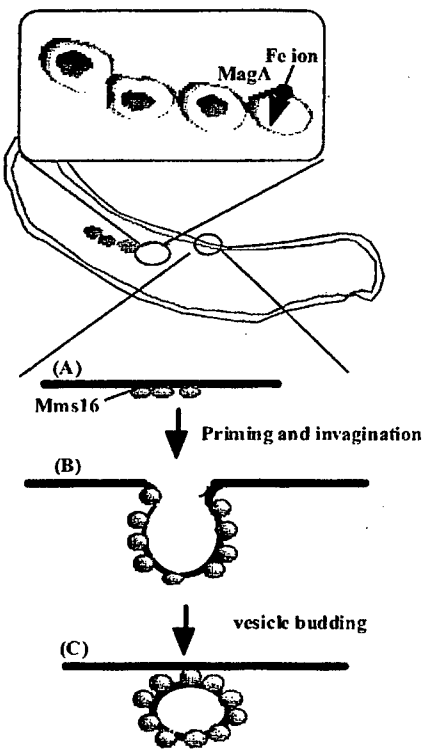

Fig. 6 Postulated mechanism of bacterial magnetic particle formation.

FUNCTIONAL BMP BASED ON MOLECULAR ARCHITECTURE

MagA, MpsA and Mms16 are all localized on the membranes of BMPs. These proteins could therefore be utilized as anchor proteins, allowing a variety of functional proteins to function on BMPs, i.e., enzymes or antibodies can be displayed on BMP membrane surfaces. For instance, employing the firefly luciferase *luc* gene, a *magA-luc* fusion gene was cloned into plasmid pRK415 and introduced into *M. magneticum* AMB-1 (Fig. 7) [32]. Extracted BMPs from recombinant AMB-1 cells expressed luciferase activity. In such a way, MagA protein acts as a workable anchor for the site-specific display of functional foreign proteins. Similarly, immunoglobulin (IgG) binding cell wall protein and proteinA,

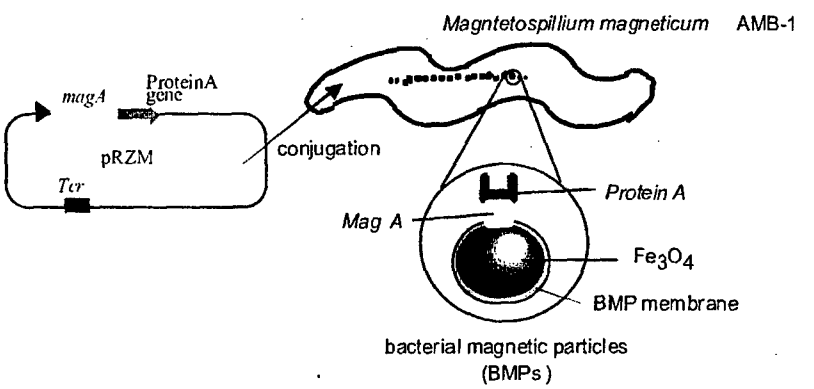

Fig. 7 Display of protein A onto bacterial magnetic particle using magA gene fusion.

were introduced to BMP membranes, using a *proteinA-magA* hybrid gene [34]. Using antibody bound proteinA-BMP complexes together with a chemiluminescence enzyme immunoassay, we have developed a rapid and highly sensitive diagnostic method for detecting human immunoglobin G (IgG) [35]. Also, the feasibility of Mms16 as an anchor molecule was established by manifesting the estrogen receptor hormone binding domain (ERHBD) on BMP membranes. ERHBD-BMP complexes function as practical receptor binding assays for estrogen-like compounds. Upscale production of functionally active antibodies or enzymes expressed on BMP membranes is effectively accomplished by fed-batch culturing techniques [36]. Further, we constructed a high copy number plasmid pUMG in AMB-1 for a more efficient display of functional foreign proteins on BMP.

APPLICATIONS OF FUNCTIONAL BMP

We have examined the functional uses of BMPs in immunoassay systems to detect various biological markers. A chief advantage of using BMPs, compared with other

conventional immunoassay methods, is the easy separation of membrane-bound and free fractions by applying a magnetic field. ProteinA-BMP complexes have viable applications in the detection of human IgG, insulin, HbA1 and glycated albumin from serum [37]. Antibodies conjugated with BMPs by chemical cross-linking methods are also valuable tools for detecting a wide variety of substances.

We have investigated various environmental pollutants, including endocrine disruptors, alkylphenol ethoxylates (APE), bisphenol A (BPA), and linear alkylbenzene sulfonates (LAS), using monoclonal antibodies immobilized on BMP and a fully automated detection system. The automated sandwich immunoassay system developed (fig. 8) comprises a reaction station, a tip rack, and an automated eight-channel pipet bearing a retractable magnet mounted close to the pipet tip and conterminous with a microtiter plate (96 wells). A single rack holds 8X3 tips for reaction. Our data show that this fully-automated system provides rapid detection (in 15 min), and efficiently determines concentrations of APE, BPA, and LAS with detection limits of 6.6 ppb, 0.023 ppt, and 35 ppt, respectively. The advantages

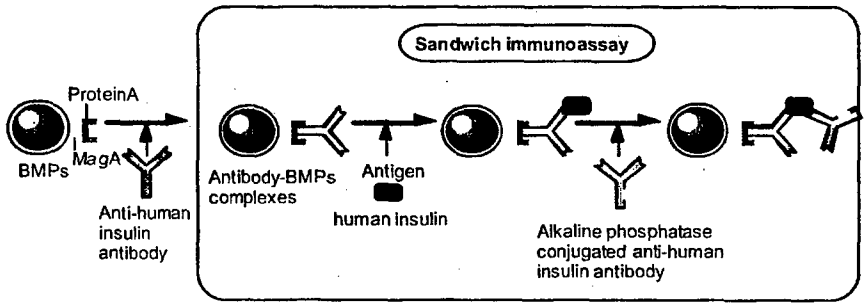

Fig. 8 Schematic diagram of sandwich immunoassay using ProteinA-BMP complexes and alkaline phosphatase-conjugated antibody.

afforded by this automated detection system should be practicable to other assay systems utilizing functional BMP.

CONCLUSION

In conclusion, BMP formation has been examined at the molecular level, although further studies are required to elucidate the whole mechanism of BMP formation and its highly controlled biomineralization process. However, recent progress in molecular biology will help enable more highly organized and systematic studies toward the elucidation of bacterial magnetite formation. In addition, improved analysis and identification of BMP specific factors will facilitate the use of biomineralization as a biomimetic technique for in vitro production of highly controlled biocrystals.

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